Characterization of monoclonal antibodies specific to wheat glutenin subunits and their correlation with quality parameters

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[1]Key Laboratory of Genetics and Biotechnology, College of Life Science, Capital Normal University, 100048 Beijing, China; [2]Institute of Vegetable Research, Shandong Academy of Agricultural Sciences, 250100 Jinan, Shandong Province, China; [3]Institute of Crop Science, National Wheat Improvement Center/The National Key Facility for Crop Gene Resources and Genetic Improvement, Chinese Academy of Agricultural Sciences (CAAS), 100081 Beijing, China; and [4]International Maize and Wheat Improvement Center (CIMMYT) China Office, c/o CAAS, 100081 Beijing, China. *Received 4 August 2008, accepted 30 September 2008.*

Li, Q., Ji, K., Zhang, Y., An, X., He, Z., Xia, X. and Yan, Y. 2009. **Characterization of monoclonal antibodies specific to wheat glutenin subunits and their correlation with quality parameters**. Can. J. Plant Sci. **89**: 11–19. Immunochemical methods are very useful in predicting the quality of wheat and differentiating alleles. In order to prepare appropriate monoclonal antibodies, HMW-GS 1Bx13 and 1By16 from spelt wheat were used as antigens to immunize BALB/C mice. Four monoclonal antibodies (mAbs) were obtained and designated 24231, 24245, 14588 and 14587, respectively. Results of Western blot showed that mAbs 24231 and 24245 prepared against 1Bx13 bound only to LMW-GS. The mAb 14588 prepared against 1By16 bound strongly to LMW-GS, but weakly to 1By and some 1Dy type HMW-GS. The mAb 14587 prepared against 1By16 bound only to 1Dx HMW-GS. The results of indirect ELISA and statistical analysis showed that correlations between mAb 24231 and development time and stability were significantly ($P<0.05$) and highly significantly ($P<0.01$) negative, respectively, whereas those of mAb 24245 with development time and extensibility were highly significantly ($P<0.01$) and significantly ($P<0.05$) negative, respectively. Significantly ($P<0.05$) and highly significantly ($P<0.01$) positive correlations were observed between mAb 14588 and stability and development time. Mean differential binding of mAb 14587 with 1Dx5^t and 1Dx2^t subunits from *Aegilops tauschii* was highly significant ($P<0.01$), suggesting that ELISA could potentially be used as an effective screening tool during direct genetic transfer of desirable glutenin subunits from *Aegilops tauschii* to hexaploid wheat.

Key words: Glutenin subunits, monoclonal antibodies, ELISA, wheat quality

Li, Q., Ji, K., Zhang, Y., An, X., He, Z., Xia, X. et Yan, Y. 2009. **Caractérisation des anticorps monoclonaux spécifiques aux sous-unités de la gluténine du blé et corrélation avec les paramètres de la qualité**. Can. J. Plant Sci. **89**: 11–19. L'immunochimie s'est avérée particulièrement utile pour prédire la qualité du blé et la différenciation des allèles. Pour préparer les anticorps monoclonaux nécessaires, les auteurs ont pris les sous-unités de la gluténine à haut poids moléculaire 1Bx13 et 1By16 de l'épeautre comme antigènes et s'en sont servi pour immuniser des souris BALB/C. Ils ont ainsi obtenu quatre anticorps monoclonaux (mAb) qu'ils ont baptisés respectivement 24231, 24245, 14588 et 14587. Les résultats de la méthode de Western indiquent que les mAb 24231 et 24245 préparés avec 1Bx13 ne se lient qu'aux sous-unités de la gluténine à faible poids moléculaire. Le mAb 14588 préparé avec 1By16 se lie fortement aux sous-unités de la gluténine à faible poids moléculaire, mais se lie peu aux sous-unités à haut poids moléculaire 1By et à certaines du type 1Dy. Le mAb 14587 préparé avec 1By16 ne se lie qu'aux sous-unités de la gluténine à haut poids moléculaire 1Dx. Les résultats obtenus avec la méthode ELISA indirecte et par analyse statistique montrent qu'il existe des corrélations négatives significatives

Abbreviations: **ELISA**, enzyme-linked immunosorbent assay; **HMW-GS**, high molecular weight glutenin subunits; **HPCE**, high performance capillary electrophoresis; **LMW-GS**, low-molecular-weight glutenin subunits; **mAbs**, monoclonal antibodies; **PBS**, phosphate-buffered saline; **PCR**, polymerase chain reaction; **RP-HPLC**, reversed-phase high performance liquid chromatography; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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($P < 0,05$) et très significatives ($P < 0,01$), respectivement, entre le mAb 24231 et le temps de développement et la qualité, alors que le mAb 24245 présente des corrélations négatives très significatives ($P < 0,01$) et significatives ($P < 0,05$), respectivement, avec le temps de développement et l'extensibilité. Les auteurs ont aussi observé des corrélations positives significatives ($P < 0,05$) et très significatives ($P < 0,01$) entre le mAb 14588 et la stabilité et le temps de développement. La liaison différentielle moyenne du mAb 14587 avec les sous-unités 1Dx5^t et 1Dx2^t d'*Aegilops tauschii* est très significative ($P < 0,01$), ce qui laisse croire que la méthode ELISA pourrait servir à la présélection lors du transfert direct de matériel génétique des sous-unités désirables de gluténine d'*Aegilops tauschii* au blé hexaploïde.

Mots clés: Sous-unités de la gluténine, anticorps monoclonaux, ELISA, qualité du blé

High molecular weight glutenin subunits (HMW-GS), including their compositions and quantity, are major determinants of bread-making quality of wheat (Payne 1987; Shewry et al. 1992). The contributions of different HMW-GS to wheat processing properties are different (Shewry and Tatham 1997). For example, subunits 1Dx5 and 1Dy10 are closely associated with good bread-making quality whereas 1Dx2 and 1Dy12 are related to poor-quality properties (Payne et al. 1979). Subunits 1Bx13 and 1By16 are also considered to determine good quality (Branlard and Dardevet 1985; Rodriguez-Quijano et al. 1990), and their contribution to bread-making quality is inferior only to 1Bx17 + 1By18 at *Glu-B1* locus (Gianibelli et al. 2001). These are not frequent subunits in bread wheat, but occur with higher frequencies in European spelt wheat, ranging from 41.5 to 87.8% (Rodriguez-Quijano et al. 1990; Caballero et al. 2001; Yan et al. 2003a, b; An et al. 2005).

Wheat quality parameters are usually assessed by Extensogram, Mixogram and Farinogram, but these instruments are expensive, and relatively large amounts of flour are required for testing. Thus, these methods are neither suitable for early stage screening of breeding lines nor for use at grain receiving stations. Methods such as SDS-PAGE (Wrigley et al. 1982; Marchylo et al. 1989), HPCE ('Werner et al. 1994; Yan et al. 2003a, b, 2004), RP-HPLC (Marchylo et al. 1988) and PCR (D'Ovidio and Anderson 1994; Varghese et al. 1996; Lafiandra et al. 1997) were usually used for HMW-GS identification. However, because of their disadvantages such as being time-consuming, having low throughput and high cost and so on, they are limited in subunits identifying and differentiating.

In recent years, enzyme-linked immunosorbent assays (ELISA) has emerged, and has been well developed. With the significant advantages of low capital and running cost, small sample requirements and simple sample handling, this method has been widely used in both wheat quality prediction and HMW-GS discrimination. One simple test for wheat quality prediction using antibodies against gliadin or glutenin subunits has been reported (Skerritt 1991a, b). The results of the assay showed that high correlations existed between antibody binding and dough-strength parameters. This assay was further optimized and used for quality analysis (Andrews et al. 1993; Hill et al. 1999). Correlations between antibody binding and wheat dough extensibility were also studied by antibodies specific for low-molecular-weight glutenin subunits (LMW-GS) (Brett et al. 1993; Andrews and Skerritt 1996). Song et al. (1998a) demonstrated that correlations between antibody binding and wheat quality varied with the type of antibodies used and wheat quality parameters. Howes et al. (1989) prepared monoclonal antibodies to distinguish γ-gliadin 45 and 42 that are linked to LMW-GS associated with good and poor pasta cooking quality, respectively. Giersch et al. (1999) obtained one monoclonal antibody binding subunit 2 more strongly than subunit 5, and an ELISA assay with the antibody was optimized for discrimination of wheat lines with the allelic pairs of 1Dx5 + 1Dy10 from those with 1Dx2 + 1Dy12.

Considerable investigations revealed that wheat-related species carried potentially useful genes for genetic improvement. *Aegilops tauschii* ($2n = 2x = 14$, DD) is the D genome donor of hexaploid wheat, and is considered the main contributor to the bread-making properties of bread wheats (Dong et al. 1991). This diploid wild species possesses not only many useful genes for resistance and tolerance to stresses, but also glutenin and other genes related to quality (Lagudah and Halloran 1988; William et al. 1993; Cox et al. 1995; Huang and Gill 2001; Yan et al. 2003b). Many of these genes have been introgressed into wheat and utilized in wheat germplasm improvement programs, either by direct hybridization to bread wheats (Gill and Raupp 1987; Hsam et al. 2001), or via tetraploid wheat × *Ae. tauschii* hybrids used in bridging crosses to hexaploid wheat, or in amphiploids (synthetic wheat). Studies showed that subunits 1Dx5, which contained one more cysteine residue than 1Dx2, contributed more to dough quality than the latter (Gupta and MacRitchie 1994). So detection of 1Dx5^t from 1Dx2^t subunits in *Aegilops tauschii* is very useful, especially in the direct genetic transfer of glutenin subunits from *Aegilops tauschii* to hexaploid wheat.

In the present work, monoclonal antibodies (mAb) against 1Bx13 and 1By16 were developed and characterized, and their applications in wheat quality prediction and specific subunit discrimination were investigated.

MATERIALS AND METHODS

Flour Samples

HMW-GS 1Bx13 and 1By16 were isolated from spelt wheat cultivar Somiedo (1, 13 + 16, 2 + 12). Thirteen

wheat cultivars with different HMW-GS compositions were used in specificity characterization of the mAbs as listed in Table 1. Twelve wheat cultivars with different quality parameters as shown in Table 2 were planted and managed under the same conditions. Twenty-two fourth back-cross lines (12 with subunit 1Dx5, 10 with subunit 1Dy2) from a Shanyou225 (HMW-GS 2+12) × Jagger (HMW-GS 5+10) and 21 *Aegilops tauschii* accessions (12 with subunits $1Dx5^t$, 9 with $1Dx2^t$) were used for subunit discrimination by monoclonal antibodies (Table 5).

Purification of HMW-GS 1Bx13 and 1By16

The glutenin fraction was extracted with 50% (vol/vol) 1-propanol containing 1% dithiothreitol (DTT) for 1hr at 50°C after pre-extraction with 70% ethyl alcohol for 30 min. The extracted components were separated on SDS-PAGE, and the gel size was 15 × 22 cm; the sample loaded was 2 mL. Electrophoresis was performed at 20 mA for 18 h at room temperature. The gel was then stained with Coomassie Brilliant Blue R-250. Subunits 1Bx13 and 1By16 were cut from the gel and were then destained with 25 mm NH_4HCO_3 containing 50% acetonitrile. After being destained, the gel pieces containing Subunits 1Bx13 or 1By16 were put in dialysis bags, and were eluted by another SDS-PAGE gel at 8 mA for 3 h. The eluted proteins were concentrated with polyethylene glycol (PEG) 6000, and then dialyzed against distilled water and phosphate-buffered saline (PBS), respectively. The purified subunits 1Bx13 and 1By16 were then collected.

Preparation of Monoclonal Antibodies

Eight- to ten-week-old female BALB/C mice were subcutaneously injected with 100 μg of antigen in 100 μL of saline emulsified with 100 μL of Freund's complete adjuvant (Sigma, St. Louis, MO). Three injections with Freund's incomplete adjuvant followed at 2- or 3-wk intervals. Serum titers of antibodies to antigens were determined by indirect ELISA 1 wk after the last injection. After 4 wk, mice with the highest serum titers received one booster injection with 100 μg of antigen in 100 μL of saline. One week later, the same mice received another booster injection just 3 d before cell fusion. On the fourth day, spleen cells from each immunized mouse were fused with SP2/0 murine myeloma cells using PEG2000 as described by Skerritt et al. (1984). After HAT (hypoxanthine, aminopterine, thymidine) selection culture of hybridoma cells, supernatants from growing cells were tested by indirect ELISA for antigen-specific antibodies. Hybridomas positive for the antigens were expanded and subcloned by the limited dilution method to ensure uniform cell populations. To produce large amounts of monoclonal antibodies, the hybridoma cells were cultured to death and supernatants containing monoclonal antibodies were collected. Another method to produce antibodies was that the hybridoma cells were injected into BALB/C mice and ascites fluids with high titers of monoclonal antibodies were collected from the mice. Isotypes of the monoclonal antibodies were determined by the mouse-type sub-typing kit (Pierce, Birmingham, AL).

ELISA Screening with Monoclonal Antibodies

HMW-GS 1Bx13 and 1By16 were prepared by the same method as used for immunization. ELISA 96 microwell plates (Costar, NY) were coated with 100 μL of 1Bx13 or 1By16 at 50 μg mL^{-1} in 50 mM carbonate buffer, pH 9.6 and incubated for 14 h at 4°C. After washing three times with PBST [PBS with 0.05% (vol/vol) Tween 20], the nonspecific binding sites on the plates were blocked with 1% gelatin (Sigma, St. Louis, MO) in 50 mM carbonate buffer, pH 9.6 and were incubated at 37°C for 30 min. The plates were again washed three times in PBST, and 100 μL $well^{-1}$ of the cell culture supernatants was added prior to 1 h incubation at 37°C. After another washing step, 100 μL of HRP-labeled goat anti-mouse IgG (Sigma, St. Louis, MO), diluted 1:10 000 in 0.1% gelatin in PBST was incubated in the wells for 1 h at 37°C. Microwells were washed four times with PBST

Table 1. High molecular weight glutenin subunits (HMW-GS) composition and wheat cultivars used for antibody screening and specificity characterization

Cultivar	HMW-GS alleles [subunits]		
Chinese Spring	*Glu-A1c* [null]	*Glu-B1b* [7+8]	*Glu-D1a* [2+12]
Zhongyou 9507	*Glu-A1a* [1]	*Glu-B1c* [7+9]	*Glu-D1d* [5+10]
Jinmai 45	*Glu-A1c* [null]	*Glu-B1c* [7+9]	*Glu-D1a* [2+12]
PI294892	*Glu-A1c* [null]	*Glu-B1g** [13*+19*]	*Glu-D1b* [3+12]
Hope	*Glu-A1a* [1]	*Glu-B1d* [6+8]	*Glu-D1d* [5+10]
PH82-2-2	*Glu-A1a* [1]	*Glu-B1e* [20]	*Glu-D1a* [2+12]
Hartog	*Glu-A1a* [1]	*Glu-B1i* [17+18]	*Glu-D1d* [5+10]
Xiaoyan 6	*Glu-A1a* [1]	*Glu-B1h* [14+15]	*Glu-D1a* [2+12]
Somiedo,	*Glu-A1a* [1]	*Glu-B1f* [13+16]	*Glu-D1a* [2+12]
Yunmai 42	*Glu-A1b* [2*]	*Glu-B1a* [7]	*Glu-D1d* [5+10]
Yannong 15	*Glu-A1a* [1]	*Glu-B1c* [7+9]	*Glu-D1c* [4+12]
Linyou 1583	*Glu-A1a* [1]	*Glu-B1d* [6+8]	*Glu-D1c* [4+12]
TRI4610/75	*Glu-A1a* [1]	*Glu-B1be* [6.1+22.1]	*Glu-D1a* [2+12]

Table 2. Quality parameters of wheat used for quality prediction[z]

Cultivar	DT	S	SV	E	R	PC
Wanmai 38	4.25	5.75	20.27	173.15	381.85	12.30
Jinghe 951	3.25	2.85	13.05	177.20	247.55	11.70
Yanyou 361	4.25	6.75	18.21	185.00	275.60	11.40
Jimai 5099	5.50	11.25	22.34	215.75	428.00	12.70
Yumai 18	1.50	1.65	11.62	197.15	121.25	10.20
Yumai 21	2.00	2.75	11.38	176.20	155.05	11.00
Shaan 253	8.50	19.50	18.68	171.80	612.50	12.40
PH82-2-2	3.35	7.75	20.88	223.20	275.10	13.00
Nongda 152	6.00	23.25	21.58	198.85	682.60	12.90
Zhongyou 9507	4.50	9.00	20.01	199.55	439.20	12.60
Zhongyou 5	2.10	1.75	11.08	150.50	105.20	11.50
Zhengmai 9023	3.50	10.50	20.86	162.00	301.95	12.00

[z]DT, development time; S, stability time; SV, sedimentation volume; E, extensibility; R, resistance; PC, protein content.

and TMB substrate [0.1 mg mL^{-1} 3,3',5,5'-tertramethyl benzidine (Amresco, Solon, OH) in 0.1 M sodium citrate buffer, pH 5.0, containing 0.03% H_2O_2] was added. After 10 min at 37°C, the reactions were stopped by adding 50 μL $well^{-1}$ of 2 M H_2SO_4. Absorbances at 450 nm were measured in a Multiscan MK3 microplate reader (Labsystems, Finland).

Specificity Characterization of Monoclonal Antibodies

Glutenin subunits were extracted from whole meals of 13 wheat cultivars, and were separated by SDS-PAGE with 10% running gel and 3.75% stacking gel at 20 mA for 4 h. The gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% (wt/vol) SDS, 5% methanol) for 40 min, and glutenin subunits were transferred to nitrocellulose membranes (Protran 0.2 μm, Pall-Gelman) in a wet blotting device at 400 mA for 9 h. After blotting, the membranes were washed three times with distilled water.

Residual binding sites on the membranes were blocked with 3% bovine serum albumin (BSA) (Amresco, Solon, OH) in PBS (50 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl) for 1 h on a rocker. After washing with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (vol/vol) Tween-20) (4×5 min), the membranes were incubated with cell culture supernatants for 1.5 h. After further washing steps (4×5 min), alkaline phosphatase-labeled anti-mouse IgG (Jackson, Newmarket, Suffolk, UK) was added at 1:1000 dilution in PBS and the membranes were incubated for 1.5 h. Another washing was performed before adding the substrate (0.033% NBT/0.0165% BCIP in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl). Specific binding of the mAb was revealed by the appearance of purple-colored bands within 5–10 min after addition of the substrate.

Analysis of Glutenin Subunits in Wheat Flour Samples

Glutenin subunits were extracted from wheat cultivars with different HMW-GS compositions. 15 mg flour from a single kernel was accurately weighted, pre-extracted with 500 μL of 70% ethanol for removing gliadins, and then glutenins were extracted with 300 μL of four solutions, respectively (50% propanol and 1% acetic acid; 10 mM HCl and 40 mM DTT; 3% SDS and 40 mM DTT; 50% propanol and 40 mM DTT) for 30 min at 65°C with regular stirring. For ELISA analysis, extracts were diluted to 200 μg mL^{-1} in 50 mM carbonate buffer, pH 9.6 and 100 μL of the diluted extracts was added to microwells and incubated for 14 h at 4°C. The wells were washed with PBST three times and nonspecific binding sites were blocked with 1% gelatin in 50 mM carbonate buffer, pH 9.6 before incubation at 37°C for 30 min. The ascites were diluted (mAb 24231 1:50; mAb 24245 1:25; mAb 14587 1:100; mAb 14588 1:75) with 0.1% gelatin in PBST, and 100 μL of each was added to the wells. Assays were performed as described above.

Allele Differentiation

Glutenin subunits were extracted from common wheat and *Aegilops tauschii* accessions according to Song et al. (1998b), in which glutenin subunits were extracted similar to the above, but after being pre-extracted with 70% ethanol, glutenin subunits were extracted using only 10 mM HCl and 40 mM DTT. The extracts were diluted to 0.2 mg mL^{-1}, and 100 μL were added to each well in the ELISA.

Statistical Analysis

T-tests for determining the significance of differences and calculation of correlation coefficients among parameters were performed.

RESULTS AND DISCUSSION

Purification of HMW-GS

The HMW-GS 1Bx13 and 1By16 subunits from spelt wheat cultivar Somiedo were separated and purified by preparative SDS-PAGE. At the step of removing bands containing the expected proteins, the entire gels were first stained, and the tagged gel strips were cut and destained so that the purified proteins could be quantified and used for immunization of mice and the mAb screening. The concentration of the purified HMW-GS 1Bx13 was 0.321 mg mL^{-1} and the total amount was 28.9 mg; the concentration and total amount of the purified 1By16 was 0.337 mg mL^{-1} and 30.3 mg, respectively.

Both purified 1Bx13 and 1By16 subunits (Fig. 1) exhibited slightly different mobilities when compared with those for total glutenin extracts from Somiedo. This probably resulted from the binding of SDS to purified subunits (Giersch et al. 1999).

Preparation and Characterization of Monoclonal Antibodies

After immunizing mice with the purified subunits, the titer of each mouse was measured, and spleen cells with the highest titers (1:128 000 for each group) were isolated and fused with myeloma cells. The supernatants for each growing cell were tested by reaction with the antigens. About 4.2% of cell supernatants for 1Bx13 and 11.2% for 1By16 secreting antibodies bound the corresponding antigens. The positive cell lines were propagated for further characterization and subcloning. Finally, four mAbs were obtained, two for 1Bx13 named mAb 24231 and mAb 24245 and two for 1By16 named mAb 14587 and mAb 14588.

The specificities of the mAbs were examined by immunobloting (Fig. 2) after SDS-PAGE separation of

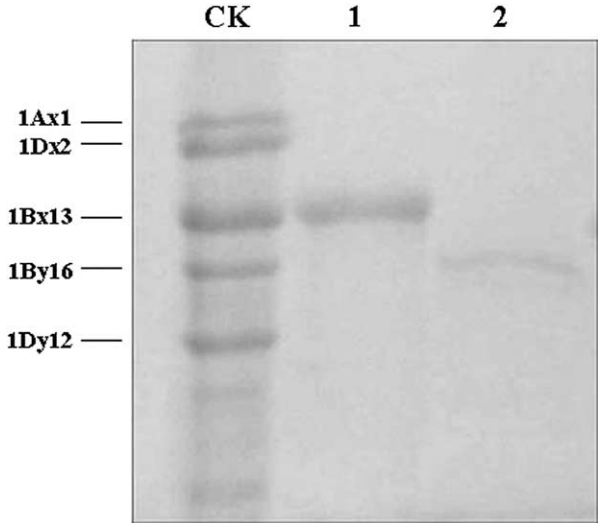

Fig. 1. SDS-PAGE of purified 1Bx13 and 1By16 subunits. CK: Somiedo. 1. purified 1Bx13. 2. purified 1By16.

total glutenin subunits from 13 wheat genotypes with different HMW-GS compositions. As shown in Fig. 2, the mAbs 24231 and 24245 bound only to LMW-GS, while the mAb 14588 bound strongly to LMW-GS and weakly to 1By and some 1Dy type HMW-GS. The mAb 14587 bound only to 1Dx HMW-GS (Fig. 2).

Apparently, there were no direct relationships between immunogen and specificities of the mAbs. Paul (2003) demonstrated that some antibodies reacted not only to the immunogen but to other correlative protein fractions, called cross-reactive antigens. In general, most antibodies bound more strongly to the immunogen than to cross-reactive antigens. Sometimes, the cross-reactive antigens take higher binding capacity than the immnnogens to some antibodies; this is an abnormal phenomenon, and the antibodies were also called abnornal antibodies. Monoclonal antibodies in this work were prepared against HMW-GS 1Bx13 and 1By16, but they bound more weakly or without binding to the immunogens in the Western blot tests, suggesting that they could be classified as abnormal antibodies. Previous work also showed that although both mAb 140820 and mAb 130612 B1# were produced by using 1Dy10 as antigen, the former reacted only to 1Bx type subunits, whereas the latter bound strongly to 1Bx and 1Dx type subunits, and only weakly to 1Dy10. In addition, mAb 110421 developed against 1Dx2 bound strongly to 1Ax, and only weakly with 1Dx2 (Giersch et al. 1999). One reason for this phenomenon might result from the high sequence homology between different glutenin subunits (Shewry et al. 1992). Another possible cause for the apparent non-specificities was due to the protein modifications during preparation, such as SDS treatment of blotted glutenins (Skerritt and Martinuzzi 1986). In this study, hybridomas cells secreting the four antibodies were screened by ELISA using purified 1Bx13 or 1By16 subunits as antigens. That is to say, in ELISA the four antibodies bound to 1Bx13 or 1By16, while in the Western blot tests, no obvious reactions were found between the four antibodies and subunits 1Bx13 or 1By16. One reason may be that the four antibodies bound much more strongly to other subunits than to 1Bx13 or 1By16. However, some difference must exist between the two methods, ELISA and Western blot. Additionally, some more common epitopes, such as cysteine, could be more immunogenic, whereas rare subunit-specific antibody-producing cells could be easily missed during cell fusion and screening.

Characteristic analysis of monoclonal antibodies showed that all the four mAbs belonged to the IgM subclass. Both 24231 and 24245 had a light λ chain, whereas 14587 and 14588 possessed a κ chain (Table 3).

Quality-antibody Binding Correlation Analysis

For glutenin extraction of wheat cultivars, four different extracting fluids were used, namely 1-propanol (50%)-acetic acid (1%) with 40 mmol L^{-1} DTT, 10 mmol L^{-1} HCl with 40 mmol L^{-1} DTT, 3% SDS with 40 mmol

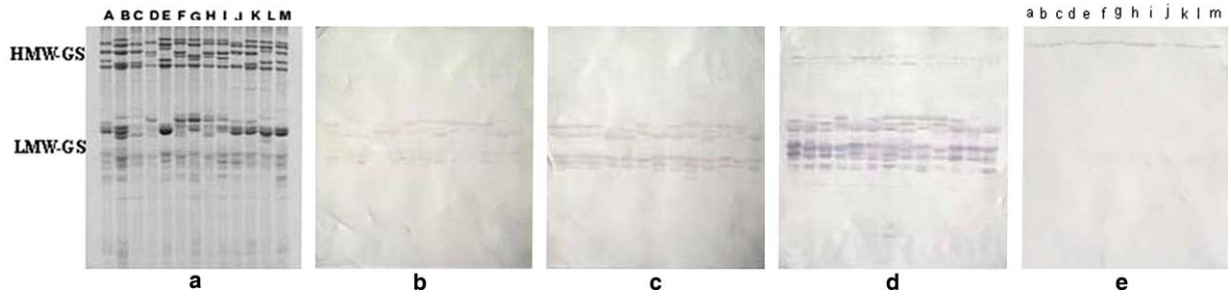

Fig. 2. SDS-PAGE separations of total glutenin sununits from 13 wheat cultivars (a). Western blotting of mAb 24231 (b), mAb 24245 (c), mAb 14588 (d) and 14587 (e) with the same genotypes. A: Chinese Spring, B: Zhongyou 9507, C: Jinmai 45, D: PI 294892, E: Hope, F: pH82-2-2, G: Hartog, H: Xiaoyan 6, I: Somiedo, J: Yunmai 42, K: Yannong 15, L: Linyou 1583, M: TRI4610/75. a: 1Dx2, b: 1Dx5, c: 1Dx3, d: 1Dx3, e: 1Dx5, f: 1Dx5, g: 1Dx2, h: 1Dx2, i: 1Dx5, j: 1Dx2, k: 1Dx4, l: 1Dx4, m: 1Dx2.

L^{-1} DTT, and 50% 1-propanol with 40 mmol L^{-1} DTT. The extraction conditions was the same, first gliadins were removed with 70% ethanol for 30 min, then glutenins were extracted using 300 μL of the above extractants for each 15 mg of kernels. ELISA absorbence values of each mAb X glutenin extract for each wheat cultivar were obtained, and the correlation coefficients between monoclonal antibody binding and flour quality parameters were calculated (Table 4).

Significant correlations between several strength-related parameters and antibody binding were observed, but varied between different antibodies, quality parameters, and extracts. The correlations between mAb24231, mAb24245 and quality parameters were much greater than those for mAb14588, and no significant correlations were found between mAb14587 and any quality parameter. Significantly negative correlation ($P < 0.05$) was observed between bindings of mAb 24231 and development time, while the correlation between bindings of mAb 24231 and stability time was highly significantly negative ($P < 0.01$). Similarly, correlations between mAb 24245 and development time, extensibility were significantly negative ($P < 0.05$). Interestingly, there was a significantly positive correlation ($P < 0.05$) between mAb 14588 and stability.

As shown in Table 4, correlations between different parameters and the mAbs were different. The development time and stability time had higher correlations with the mAbs, then with the extensibility. No significant correlations were found between antibodies and sedimentation volume, maximum resistance or protein content in this study. Results for the different extraction fluids indicated that 50% 1-propanol with 1% acetic acid was better than HCl, SDS or 1-propanol extracts, but there existed some variations between different antibodies. For example, the highest correlation was obtained for binding of mAb24231 and quality parameters when glutenin was extracted with 3% SDS, whereas the same results for mAb14588 occurred when glutenin were extracted with 10mM HCl.

Detecting for wheat quality using immunochemical method was reported previously. Skerritt (1991a, b) detected quality properties of different wheat materials using different antibodies, screened and confirmed suitable work conditions for ELISA, and developed one testing method to fit for the local fact. Studies showed that specific antibodies could detect wheat dough properties effectively (Skerritt 1991a, b; Andrews et al. 1993). Theoretically, each of the quality properties could be detected by selecting appropriate antibodies. However, results of the few reports indicated that it was just effective to use antibodies to detect wheat strength related properties, such as development time, stability time, extensibility, resistance etc. In this study, the higher correlation coefficients appeared between some monoclonal antibodies and development time, stability time, extensibility and maximal resistance, respectively. This was consistent with the results reported before. Correlations between antibody binding and protein content were lower in this study, which was in agreement with the results of Skerritt (1991b), but differed from the report of Song et al. (1998a), in which correlations between antibody binding and protein content were high and significant. Correlations between antibody binding and sedimentation volume were also very low in this study, similar to the result of Song et al. (1998a). Both Skerritt (1991a) and Song et al. (1998a) suggested that the abilities of different antibody to detect wheat quality properties were different, and some antibodies had no significant correlations with some quality parameters. In this work, mAb 14587 was highly specific, binding only to HMW-GS 1Dx, but with

Table 3. Specificity and isotype of the four monoclonal antibodies

Immunogen	Antibody	Specific for glutenin subunits	Isotype
1Bx13	24231	LMW-GS	IgM, λ
	24245	LMW-GS	IgM, λ
1By16	14587	1Dx	IgM, kappa
	14588	LMW-GS, 1By, 1Dy	IgM, kappa

Table 4. Correlation coefficients between monoclonal antibody absorbance values and flour quality parameters using four protein extracting conditions[z]

mAbs	Extracts	DT	S	SSDS	E	R	PC
24231	A	−0.583*	−0.499	−0.505	0.099	−0.533	−0.457
	B	0.221	0.313	0.201	0.452	0.161	0.283
	C	0.103	−0.723**	0.195	0.088	0.027	0.263
	D	−0.43	−0.482	−0.253	−0.5	−0.353	−0.094
24245	A	−0.775**	−0.534	−0.416	0.102	−0.538	−0.466
	B	−0.063	−0.132	0.425	−0.161	0.111	0.09
	C	0.454	0.403	0.283	0.054	0.405	0.235
	D	−0.47	−0.453	−0.296	−0.686*	−0.299	−0.372
14587	A	0.214	0.188	0.453	0.127	0.343	0.083
	B	−0.363	−0.395	0.24	0.485	−0.318	0.269
	C	−0.194	−0.306	−0.015	−0.266	−0.452	−0.094
	D	0.153	0.159	0.165	−0.363	0.224	0.085
14588	A	−0.225	−0.284	0.071	−0.072	−0.255	−0.26
	B	0.694**	0.625*	0.266	0.131	0.431	0.392
	C	0.237	0.215	−0.182	0.266	−0.041	0.375
	D	0.149	0.115	−0.135	−0.512	0.084	0.076

[z]DT, development time; S, stability; SSDS, SDS sedimentation volume; E, extendibility; R, resistance; PC, protein content; A, 1-propanol (50%)-acetic acid (1%)+40mmol L^{-1} DTT; B, 10 mmol L^{-1} HCl+40 mmol L^{-1} DTT; C: 3% SDS+40 mmol L^{-1} DTT; D: 50% 1-propanol+40 mmol L^{-1} DTT.
*, ** $P<0.05$ and $P<0.01$, respectively, Student's *t*-test.

no significant correlations with any of the parameters detected.

Correlations between mAb 24231 and development time or sedimentation volume, between mAb 24245 and development time or extensibility were all significantly negative, while most of the antibodies reported before (Skerritt 1991b; Song et al. 1998a) were significantly positive to wheat quality parameters. This might result from the different specificity of two antibodies, which only bound to LMW-GS, without binding to HMW-GS. However, the antibodies reported before, which showed significantly positive correlations with wheat quality properties, bound only to some HMW-GS or not only to LMW-GS and gliadins but to HMW-GS (Skerritt and Underwood 1986; Donovan et al. 1989; Skerritt and Robson 1990; Song et al. 1997, 1998c). In this study, the antibody mAb 14588, binding not only to HMW-GS, but to some LMW-GS, also had significantly positive correlations with both wheat quality parameters development time and sedimentation volume.

Correlations between antibody binding and wheat quality properties were also affected by glutenin extractants. Studies by Song et al. (1998a) showed that extractant A [1-propanol (50%)-acetic acid (1%)+40 mmol L^{-1} DTT] was better than B (10 mmol L^{-1} HCl+40 mmol L^{-1} DTT) and C (3% SDS+40 mmol L^{-1} DTT). The results from Skerritt (1991a) showed that the SDS-DTT was the best extractant with the strongest cook sample yielding the highest ELISA absorbances. Our results showed that the best extractant varied in different mAbs. For instance, for mAb 24231 and mAb 24245, extractant A was the best, but for mAb 14587 and mAb 14588, extractant B appeared to be best.

However, correlations between antibody binding and wheat quality parameters found here were not enough and mostly poor. In fact, many quality parameters can be measured for estimating end-use quality in wheat; however, only six main parameters were used in this work because of limits of time and energy. It is possible that higher correlations exist between monoclonal antibodies and other non-analyzed parameters.

Discrimination of Allelic Pairs of HMW-GS

In order to understand their application potential for discriminating different HMW-GS, four monoclonal antibodies developed were assessed by the back-cross lines from bread cultivar Shanyou 225 (2+12) × Jagger (5+10) and the *Aegilops tauschii* accessions carrying the same two alleles, respectively. The results showed that all mAbs could not discriminate 1Dx5 and 1Dx2 subunits of hexaploid wheat. This was consistent with the results of Mills et al. (2000), in which a monoclonal antibody developed to a synthetic peptide of HMW subunit 1Dx5 was found bound strongly to the synthetic peptide based on the cognate sequence of HMW subunit 1Dx2, while recognizing the immunizing peptide by ELISA only poorly. The reason may be that the gene sequences of 1Dx5 and Dx2 were highly identical (Anderson et al. 1989), so it was difficult to differentiate them by these mAbs.

When testing *Aegilops tauschii* accessions, mAb14587 could differentiate $1Dx5^t$ and $1Dx2^t$ subunits well (Table 5). As shown in Table 5, bindings of mAb14587 with *Aegilops tauschii* accessions containing subunit $1Dx5^t$ were lower than those with $1Dx2^t$. Results of statistical analysis showed that the difference was significant ($P<0.01$). These results suggest that

Table 5. Bindings of 21 *Aegilops tauschii* accessions to mAb 14587

Aegilops tauschii accessions	HMW-GS 1Dx composition	Absorbance at A450
AT87[z]	$1D \times 5^t$	0.403 ± 0.011
AT58	$1D \times 5^t$	0.477 ± 0.014
AT11	$1D \times 5^t$	0.482 ± 0.033
AT176	$1D \times 5^t$	0.512 ± 0.014
AT51	$1D \times 5^t$	0.521 ± 0.017
AT193	$1D \times 5^t$	0.534 ± 0.009
AT26	$1D \times 5^t$	0.545 ± 0.013
AT47	$1D \times 5^t$	0.564 ± 0.008
AT18	$1D \times 5^t$	0.578 ± 0.026
AT59	$1D \times 5^t$	0.599 ± 0.016
AT198	$1D \times 5^t$	0.614 ± 0.007
AT158	$1D \times 5^t$	0.632 ± 0.012
AT182	$1D \times 2^t$	0.651 ± 0.011
AT21	$1D \times 2^t$	0.656 ± 0.005
AT147	$1D \times 2^t$	0.706 ± 0.03
AT143	$1D \times 2^t$	0.713 ± 0.015
AT113	$1D \times 2^t$	0.723 ± 0.005
AT185	$1D \times 2^t$	0.768 ± 0.006
AT22	$1D \times 2^t$	0.780 ± 0.015
AT13	$1D \times 2^t$	0.780 ± 0.02
AT130	$1D \times 2^t$	0.920 ± 0.023

[z]AT refers to the germplam collection held at Capital Normal University.

ELISA could potentially be used as an effective screening tool during direct genetic transfer of desirable glutenin subunits from *Aegilops tauschii* to hexaploid wheat.

In our judgment, the different results for testing 1Dx5 and 1Dx2 in common wheats and $1Dx5^t$ and $1Dx2^t$ in *Aegilops tauschii* might result from the effects of cross reactions with other proteins. Common wheats were hexaploid ($2n = 6x = 42$, AABBDD), and contained more storage proteins with similar immunochemical properties, especially for LMW-GS, which might interfere with the testing results. In our experiments, when the concentration of glutenin extracts was very high, the mAb14587 could also bind with LMW-GS, but much more weakly than those with 1Dx HMW-GS. However, *Aegilops tauschii* was diploid ($2n = 2x = 14$, DD) and only had a *Glu-D3* locus. Therefore, effects of LMW-GS on ELISA should be much less than those in common wheats. Another reason might be immunochemical differences between 1Dx5 and 1Dx2 in common wheats and $1Dx5^t$ and $1Dx2^t$ in *Aegilops tauschii*. It was found that there existed some differences between 1Dx5 and $1Dx5^t$ as well as 1Dx2 and $1Dx2^t$ in relative mobilities and gene structures (Yan et al. 2003b; Zhang et al. 2008). It was possible that greater immunochemical differences were present between $1Dx5^t$ and $1Dx2^t$ in *Aegilops tauschii* and, therefore, they could be discriminated by mAb14587.

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An, X., Yan, Y. M., Xiao, Y. H., Li, Q. Y., Hsam, S. L. K. and Zeller, F. J. 2005. Genetic diversity of European spelt wheat (*Triticum spelta* L.) revealed by glutenin subunit variations at *Glu-1* and *Glu-3* loci. Euphytica **146**: 193–201.

Anderson, O. D., Greene, F. C., Yip, R. E., Halford, N. G., Shewry, P. R. and Malpica-Romero, J. M. 1989. Nucleotide sequences of the two high-molecular- weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. Nucleic Acids Res. **17**: 461–462.

Andrews, J. L., Blundell, M. J. and Skerritt, J. H. 1993. A simple antibody-based test for dough strength. III. Further simplification and collaborative evaluation for wheat quality screening. Cereal Chem. **70**: 241–246.

Andrews, J. L. and Skerritt, J. H. 1996. Wheat dough extensibility screening using a two-site enzyme-linked immunosorbent assay (ELISA) with antibodies to low molecular weight glutenin subunits. Cereal Chem. **73**: 650–657.

Branlard, G. and Dardevet, M. 1985. Diversity of grain proteins and bread wheat quality. II. Correlation between high-molecular-weight subunits of glutenin and flour quality characteristics. J. Cereal Sci. **3**: 345–354.

Brett, G. M., Mills, E. N. C., Tatham, A. S., Fido, R. J., Shewry, P. R. and Morgan, M. R. A. 1993. Immunochemical identification of LMW subunits of glutenin associated with bread-making quality of wheat flours. Theor. Appl. Genet. **86**: 442–448.

Caballero, L., Martin, L. M. and Alvarez, J. B. 2001. Allelic variation of the HMW glutenin subunits in Spanish accessions of spelt wheat (*Triticum aestivum* ssp. *spelta* L. em. Thell). Theor. Appl. Genet. **103**: 124–128.

Cox, T. S., Sears, R. G., Bequette, R. K. and Martin, T. J. 1995. Germplasm enhancement in winter wheat × *Triticum tauschii* backcross populations. Crop Sci. **35**: 913–919.

D'Ovidio, R. and Anderson, O. D. 1994. PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread-making quality. Theor. Appl. Genet. **88**: 759–763.

Dong, H., Cox, T. S., Sears, R. G. and Lookhart, G. L. 1991. High molecular weight glutenin genes: Effects on quality in wheat. Crop Sci. **31**: 974–979.

Donovan, G. R., Skerritt, J. H. and Castle, S. L. 1989. Monoclonal antibodies used to characterize cDNA clones expressing specific wheat endosperm proteins. J. Cereal Sci. **9**: 97–111.

Gianibelli, M. C., Masci, S., Larroque, O. R., MacRitchie, F. and Wrigley, C. W. 2001. Biochemical, genetic and molecular characterization of wheat glutenin and its component subunit. Cereal Chem. **78**: 635–646.

Giersch, T. M., Hill, A. S. and Skerritt, J. H. 1999. Development of a panel of specific monoclonal antibodies to high molecular weight glutenin subunits and their application in genetic screening. Cereal Chem. **76**: 380–388.

Gill, B. S. and Raupp, W. J. 1987. Direct genetic transfer from *Aegilops squarrosa* L. to hexaploid wheat. Crop Sci. **27**: 445–450.

Gupta, R. B. and MacRitchie, F. 1994. Allelic variation at glutenin subunit and gliadin loci, Glu-1, Glu-3 and Gli-1 of

common wheats. II. Biochemical basis of the allelic effects on dough properties. J. Cereal Sci. **19**: 19–29.

Hill, A. S., Giersch, T. M., Loh, C. S. and Skerritt, J. H. 1999. Immunoassay for wheat processing quality: Utilization of a Sandwich assay incorporating an immobilized single-chain fragment. J. Agric. Food Chem. **47**: 4484–4490.

Howes, N. K., Kovacs, M. I., Leisle, D., Dawood, M. R. and Bushuk, W. 1989. Screening of durum wheats for pasta-making quality with monoclonal antibodies for gliadin 45. Genome **32**: 1096–1099.

Hsam, S. L. K., Kieffer, R. and Zeller, F. J. 2001. Significance of *Aegilops tauschii* glutenin genes on breadmaking properties of wheat. Cereal Chem. **78**: 521–525.

Huang, L. and Gill, B. S. 2001. An RGA-like marker detects all known *Lr21* leaf rust resistance gene family members in *Aegilops tauschii* and wheat. Theor. Appl. Genet. **103**: 1007–1013.

Lafiandra, D., Tucci, G. F., Pavoni, A., Turchetta, T. and Margiotta, T. 1997. PCR analysis of x- and y-type genes present at the complex *Glu-D1d* locus in durum and bread wheat. Theor. Appl. Genet. **94**: 235–240.

Lagudah, E. S. and Halloran, G. M. 1988. Phylogenetic relationships of *Triticum tauschii* the D genome donor to hexaploid wheat 1.Variation in HMW subunits of glutenin and gliadins. Theor. Appl. Genet. **75**: 592–598.

Marchylo, B. A., Handel, K. A. and Mellish, V. J. 1989. Fast horizontal sodium dodecyl sulfate polyacrylamide electrophoresis for rapid wheat cultivar identification and analysis of high molecular weight glutenin subunits. Cereal Chem. **66**: 186–192.

Marchylo, B. A., Hatcher, D. W. and Kruger, J. E. 1988. Identification of wheat cultivars by reversed-phase high-performance liquid chromatography of storage proteins. Cereal Chem. **65**: 28–40.

Mills, E. N. C., Field, J. M., Kauffman, J. A., Tatham, A. S., Shewry, P. R., Morgan, M. R. A. 2000. Characterization of a monoclonal antibody specific for HMW subunits of glutenin and its use to investigate glutenin polymers. J. Agric. Food Chem. **48**: 611–617.

Paul, W. E. (ed.) and Wu, Y. (ed. and translator) 2003. Fundamental immunology. Science Press, Beijing, China [in Chinese].

Payne, P. I. 1987. Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. Annu. Rev. Plant Physiol. **38**: 141–153.

Payne, P. I., Corfield, K. G. and Blackmann, J. A. 1979. Identification of high-molecular- weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theor. Appl. Genet. **55**: 153–159.

Rodriguez-Quijano, M., Vazquez, J. F. and Carrillo, J. M. 1990. Variation of high-molecular-weight glutenin subunits in Spanish landraces of *Triticum aestivum* ssp. *vulgare* and ssp. *spelta*. J. Genet. Breed. **44**: 121–126.

Shewry, P. R., Halford, N. G. and Tatham, A. S. 1992. The high molecular weight subunit of wheat glutenin. J. Cereal Sci. **15**: 105–120.

Shewry, P. R. and Tatham, A. S. 1997. Biotechnology of wheat quality. J. Sci. Food Agric. **73**: 397–406.

Skerritt, J. H. 1991a. A simple antibody-based test for dough strength. I. Development of method and choice of antibodies. Cereal Chem. **68**: 467–474.

Skerritt, J. H. 1991b. A simple antibody-based test for dough strength. II. Genotype and environmental effects. Cereal Chem. **68**: 475–481.

Skerritt, J. H. and Martinuzzi, O. 1986. Effects of solid phase and antigen solvent on the binding and immunoassay of water soluble flour proteins. J. Immunol. Meth. **88**: 217–224.

Skerritt, J. H. and Robson, L. G. 1990. Wheat low molecular weight glutenin subunits. Structural relationship to other gluten proteins analyzed using specific antibodies. Cereal Chem. **67**: 250–257.

Skerritt, J. H., Smith, R. A., Wrigley, C. W. and Underwood, P. A. 1984. Monoclonal antibodies to gliadin proteins used to examine cereal grain protein homologies. J. Cereal Sci. **2**: 215–224.

Skerritt, J. H. and Underwood, P. A. 1986. Specificity characteristics of monoclonal antibodies to wheat grain storage proteins. Biochim. Biophys. Acta **874**: 245–254.

Song, X., Cai, Q., Gao, W., Liu, G. and Huang, T. 1998a. Immunochemical assay for quality and immuno-logical homology of storage proteins in different closely related genera and species of wheat. Acta Bot. Sin. **40**: 527–533.

Song, X., Gao W., Liu, G. and Huang, T. 1997. Preparation of monoclonal antibodies to high-molecular-weight glutenin subunit (HMW-GS) of wheat endosperm storge proteins. J. Agric. Biotechnol. **5**: 25–34.

Song, X., Gao, W., Liu, G. and Huang, T. 1998b. Application of monoclonal antibodies to high-molecular-weight glutenin subunit (HMW-GS) 1Dy10 of wheat endosperm storage protein in quality breeding. Acta Agron. Sin. **24**: 298–303.

Song, X., Gao, W., Lu, D., Huang, T. and Liu, G. 1998c. Preparation of Antisera to high-molecular-weight glutenin subunits (HMW-GS) of wheat endosperm storage protein. J. China Agric. Univ. **3**: 1–6.

Varghese, J. P., Struss, D. and Kazman, M. E. 1996. Rapid screening of selected European winter wheat varieties and segregating populations for the *Glu-D1d* allele using PCR. Plant Breed. **115**: 451–454.

Werner, W. E., Wiktorowicz, J. E. and Kasarda, D. D. 1994. Wheat varietal identification by capillary electrophoresis of gliadins and high molecular weight glutenin subunits. Cereal Chem. **71**: 397–402.

William, M. D. H. M., Pena, R. J. and Mujeeb Kazi, A. 1993. Seed protein and isozyme variations in *Triticum tauschii* (*Aegilops squarrosa*). Theor. Appl. Genet. **87**: 257–263.

Wrigley, C. W., Autran, J. C. and Bushuk, W. 1982. Identification of cereal varieties by gel electrophoresis of the grain proteins. Adv. Cereal Sci. Technol. **5**: 211–259.

Yan, Y., Hsam, S. L. K., Yu, J., Jiang, Y., Ohtsuka, I. and Zeller, F. J. 2003a. HMW and LMW glutenin alleles among putative tetraploid and hexaploid European spelt wheat (*Triticum spelta* L.) progenitors. Theor. Appl. Genet. **107**: 1321–1330.

Yan, Y., Hsam, S. L. K, Yu, J., Jiang Y. and Zeller, F. J. 2003b. Allelic variation of the HMW glutenin subunits in *Aegilops tauschii* accessions detected by sodium dodecyl sulphate (SDS-PAGE), acid polyacrlamide gel (A-PAGE) and capillary electrophoresis. Euphytica **130**: 377–385.

Yan, Y., Zheng, J., Xiao, Y., Yu, J., Hu, Y., Cai, M., Hsam, S. L. K. and Zeller, F. J. 2004. Identification and molecular analysis of a y-type HMW glutenin subunit gene from *Glu-1D*^{t} locus of *Aegilops tauschii*. Theor. Appl. Genet. **108**: 1349–358.

Zhang, Y., Li, X., Wang, A., An, X., Zhang, Qian., Pei, Y., Gao,L., Ma, Wujun, Appels, R. and Yan, Y. 2008. Novel x-type high-molecular-weight glutenin genes from *Aegilops tauschii* and their implications on the wheat origin and evolution mechanism of *Glu-D1-1* proteins. Genetics **178**: 23–33.